PHYSIOLOGICAL AND GENETIC STUDIES WITH TRYPSIN INHIBITOR OF CORN (ZEA mays L.)

by

SIZI ZUBANYEA MORRIS

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To my most loving and understanding father,

J. Subah Morris, Sr.

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INTRODUCTION

Maize has become an increasingly important cereal crop in world food and commercial channels. It's importance stems from its use as a major source of animal feed as well as a primary food for human consumption. In some particularly large geographic regions of the world, eg. India, Pakistan, Mexico, and certain portions of East Africa, it is the staple food or main food source. Such people depend on maize not only for relief from hunger, but also as a source of most of the proteins needed for the body. Thus the concern for human nutrition has led to a great deal of attention given to improvement of maize protein quality.

The most significant quality change in maize has been brought on by the discovery of the opaque-2 mutant gene which increases the levels of lysine and tryptophan, two limiting amino acids in maize. Before the discovery of opaque-2, maize lines were nutritionally deficient from the protein standpoint which resulted from low levels of lysine and tryptophan.

The opaque-2 mutant gene has now been successfully introduced into lines of maize, but work still continues on improving overall protein quality. Studies with the opaque-2 gene in maize have shown that lines carrying the opaque-2 gene, not only double their lysine and tryptophan contents but also increase another chemical component of the seed, trypsin inhibitor.

The concept of corn trypsin inhibitor is relatively new and has received very little study, but the occurrence of trypsin inhibitor in other plant species like soybeans (Glycine max) and mungbeans (Phaseolus aureus) has been studied extensively for some time. The generated interest in corn trypsin inhibitor probably originates from the results of studies on trypsin inhibitors of soybeans and other crops on growth of rats and chicks. In these studies, trypsin was found to cause growth retardation and pancreatic hypertrophy. It would therefore seem quite logical and justifiable to investigate whether the same phenomenon holds true for corn trypsin inhibitors and what possible consequences this would have on maize as a crop for human consumption.

Mitchell et al. (39) have presented research results with corn trypsin inhibitor that suggests trypsin inhibitor in corn is quite safe for animal and human consumption since high concentrations in rat diets did not significantly affect growth or any other noticeable character. However, from an agronomic point it has been postulated that lines carrying the opaque-2 gene germinate more slowly than normal lines.

The present study with trypsin inhibitor includes both physiological and genetic aspects of trypsin inhibitor in maize since such information would be vital if the need should arise for altering trypsin inhibitor content of corn. The physiological study deals with the level changes of trypsin inhibitor from germination through physiological maturity in eight lines

of corn. The genetic study estimates heritability, gene effects and possible number of genes involved in the character trypsin inhibitor in three crosses of corn inbreds.

For sampling convenience, the physiological study has been subdivided into an environmental chamber study designed to trace trypsin inhibitor level changes from germination to seedling establishment, a greenhouse study that traces trypsin inhibitor level changes from seedling establishment, through vegetative development on to flowering, and a field study that traces trypsin inhibitor level changes from flowering to physiological maturity.

The genetic study uses the means and variances of the P_1, P_2, F_1, F_2, BC_1 and BC_2 of each cross to estimate the desired parameters. All measurements taken for this study were at physiological maturity.

LITERATURE REVIEW

The deficiency of the essential amino acids, lysine and tryptophan, in corn (Zea mays L.) was first reported by Osborne et al. (41). Harvey and Oaks found this to be true in their classification of cereal endosperm storage proteins when they discovered that zein and glutelin proteins differed in amino acid composition. Zein, which makes up a greater portion of the total protein, was deficient in lysine and tryptophan and to a lesser extent in arginine, histidine and methionine (25).

The extensive use of maize as a staple food source for human consumption and its high priority as a primary animal feed for monogastric animals, coupled with progress made in the field of nutrition, stressed the necessity for improving the amino acid composition of maize. This breakthrough was accomplished in 1964 (18, 40) by the discovery that the opaque-2 recessive mutant gene alters the amino acid profile of maize endosperm and results in a higher lysine concentration (40). The opaque-2 gene was first discovered in 1937 by Singleton and Jones (18) but the only use for it then was as a phenotypically recognizable marker in the short arm of chromosome seven. It wasn't until 1963 when three scientists at Purdue University discovered the biochemical effect of the opaque-2 gene that it received renewed attention.

Nelson, Mertz, and Bates (40) found that endosperm protein of maize homozygous for the opaque-2 gene contained 70% more

lysine and more tryptophan than normal maize endosperm. The high lysine contents of these opaque-2 lines were attributed to two factors; a higher lysine content of endosperm and a lesser zein production in the endosperms of opaque-2 lines.

The opaque-2 gene in corn not only doubles the lysine and tryptophan contents but also doubles the level of trypsin inhibitor (23, 39). Trypsin inhibitor is a naturally occurring protein (4, 15, 23, 38) belonging to the general group of protease inhibitors (3, 4, 23, 33) which are widely distributed among those very plants which contribute an important source of dietary protein throughout the world (33). Liener (33) has defined protease inhibitors as substances with the ability to inhibit proteolytic activity.

The presence of an inhibitor of trypsin in plants was first recognized in 1938 by Read and Haas (33). Most research concerning protease inhibitors has been done with soybean trypsin inhibitors. Study results led to the realization that protease inhibitors might be of significance in plant foodstuffs, and eventually stimulated the search for similar factors in other plant material.

Plant protease inhibitors studied to data have been found in seeds of various plants but they are also found in the stems, cotyledons and leaves of others (3, 22, 33, 37). Soybean and kidney beans have a greater concentration of the inhibitors in the outer part of the cotyledons; Mungbeans have high inhibitor levels in leaves and cotyledons but very low levels in the stem. In the Double beans and Field beans, the protease

inhibitors are distributed throughout the germinating seed and growing plant. Corn contains high levels of trypsin inhibitor in the endosperm of the seed (23, 37). Some common crops in which inhibitors have been found in addition to the few mentioned above are oats, wheat, barley, rice, sweet potato, peanuts and lima beans.

Birk et al. (6) nutritionally rated protease inhibitors along with amylase and urease as the most important biologically active proteins present in soybean meal. Hymowitz and Hadley (27) considered trypsin inhibitor in raw soybeans as one of the factors responsible for the inferiority of unheated soybean meal compared to properly heated soybean meal. Desikachar and De (15) had earlier discovered that cooking improved the nutritive value of the protein of soybeans but without recognizing the role of trypsin inhibitor.

Germination has been characterized by the mobilization of protein reserves from the storage tissues and the transfer of solubilized derivatives to the growing embryo axis (2, 5, 11, 43). Workers are agreed that the metabolism of these reserve proteins depends entirely on the appearance of endopeptidase activity (2, 11, 43), but there is some disagreement concerning the source of these enzymes. Some claim the enzymes are contained in the protein bodies of dormant seeds and are released with the onset of germination (25, 26, 43). This view is contested by others who believe the enzymes are synthesized de-novo after the initiation of germination (5, 11). Nevertheless, irrespective of the source, proteolytic enzyme activity has

been shown to increase to a maximum as germination progresses up to the 5th to 8th day and then declines to the 12th day (5, 9, 43, 52). This maximum point of enzyme activity occurs when the maximum breakdown of reserve proteins takes place.

Trypsin inhibitors have also been found in high concentrations in dormant as well as germinating seeds (9, 23, 33, 38). Their inhibitory activity has been measured in a variety of crops (33). In maize, trypsin inhibitory activity was found to be highest in the starchy endosperm of dry seeds (23, 36), and to vary within strains at different growth stages (23). In horsegram and mothbeans, trypsin inhibitory activity was found to decrease 16% and 40%, respectively, upon germination for 72 hours (49). In ungerminated pea seeds, trypsin inhibitory activity was high but decreased upon germination (26). Baumgartner and Chrispeels (3) and several other workers observed that the decrease in trypsin inhibitory activity during germination was accompanied by a corresponding rise in endopeptidase activity, thus suggesting the inhibitors as endopeptidase activity regulators. Hobday et al. (26), contrastingly suggest it improbable for trypsin inhibitory activity to regulate endopeptidase activity during germination when the proteases are located within the protein bodies and the majority of the trypsin inhibitors found outside the protein bodies. These conflicting views have succeeded in opening up a new line of inquiry as to the role of trypsin inhibitors in plants.

Mikola and Suolinna (37), on the assumption that inhibitors are primarily directed against other proteolytic enzymes and

the inhibition of trypsin is only a side effect, have proposed several hypotheses to explain the presence of trypsin inhibitors in seeds: a) inhibitors affect endogenous seed proteinases in addition to trypsin and so protect the seed from autolysis during the resting stage; b) Trypsin inhibitors inhibit microbial proteinases and function to protect the seed from proteolytic micro-organisms; c) for plants like barley for which the above hypotheses do not apply, trypsin inhibitors are essential for the endozooic dispersal of seeds - i.e., the presence of trypsin inhibitors in high concentrations in such seeds increases the percentage of seeds passing through the alimentary canal of animals that eat fruits or whole plants and excrete viable seeds.

Mitchell et al. (38) hypothesized trypsin inhibitors may function by reducing fungal growth during seed germination or during other periods when seeds are moist enough to support fungal growth. Pusztai conjectures that high cystine content of trypsin inhibitors might be exploited by plants as cystine and/or sulfur depot proteins (42).

Nutritionally, trypsin inhibitors have been deemed responsible for reducing the digestibility of proteins by inhibiting tryptic activity (13). Rachis (1965) and Kakade et al. (1973) reported that soybean trypsin inhibitors in the diets of rats depressed growth and caused pancreatic hypertrophy (39). Probable cause of this low nutritive value was supposedly due to inhibitors decreasing the degree of availability or rate of release of methionine, the limiting amino acid in soybean.

Desikachar and De (15) found that cooking soybeans increased the nutritive value of its protein by increasing the availability of cystine and methionine. Conclusion was that the role of inhibitors in deciding the nutritive value of raw soybeans is not in deminishing the degree of availability or release of methionine but rather may function as an antigrowth factor affecting the usefulness of proteins in general. The above conclusion was supported by Ham et al. (7, 15) in a study of proteolytic inhibitors causing growth retardation in chicks fed on a well-balanced diet.

Conducting similar experiments with isolated corn trypsin inhibitor, Mitchell et al. (38), added trypsin inhibitor to rat diets. There was no decrease in growth, nor did pancreatic hypertrophy occur.

MATERIAL AND METHODS

A. Physiological Study

a. Environmental chamber study. Ten seeds of each of five corn inbred lines - H28, SD10, K724, K41 and K201G and three heterogeneous sources - O_2-S, O_2-H, and FL_2, were imbedded in a 12 x 11 inch metal tray half-filled with moist vermaculite. Each tray contained eight rows of seeds, one row per source, and there was a total of 10 trays in all. Trays were placed in an environmental chamber at 70°F run under a regime of eight-hours of light and sixteen hours of darkness.

Sampling for trypsin inhibitor level assay was by random removal of one tray each day. A control sample was taken before the seeds were placed in the trays. Samples were washed to remove the vermaculite and then ovendried under vacuum at 50°C for 18-20 hours. Samples taken were whole seedling samples (roots + shoots).

b. Greenhouse study. The same eight genetic sources of corn used in the germination experiment were also used for this study. A total of 12 pots per entry, three seeds per pot were planted for the experiment. Pots were marked and arranged on benches in the greenhouse. Sampling began five days after planting when seedlings were fully emerged, and subsequent samples were taken on the 7th, 11th, 14th, 16th, 26th, 32nd, 42nd, 53rd, and 63rd days after planting.

Due to the small size of seedlings, the first to fifth

samples taken were whole plant samples, and all other samples thereafter were for stem and leaf. Three plants per sample were taken during the first seven sampling periods and two for the last three samplings. Sampling was by randomly digging up a plant from three of the twelve pots of each genotype but in somewhat of a restricted manner so as to thin out the plants in each pot. Sampling was discontinued on the 63rd day when most genotypes began to tassel. Stem samples were obtained from the middle internodes whereas leaf samples were derived from a leaf composite of all the plants sampled.

c. Field study. The same eight sources used for the environmental chamber and the greenhouse studies were used for the field experiment. Each genotype was planted in four row plots 20-feet long, replicated three times per location; plants were spaced one foot apart in rows three feet apart. Two locations, Ashland (Riley County) and Manhattan North Agronomy Farm were used. Self-pollinations were made in each plot.

Four sampling periods were used for this portion of the study, ans were designed to approximate the blister, dough, dent and physiological maturity stages. Sampling involved randomly selecting self-pollinated ears from any of the inner two rows of each plot of the three replications at each location. Samples were immediately taken to the laboratory and processed for trypsin inhibitor assay.

B. Genetic Study

Selection of corn sources for the genetic study was based

on F_1 crosses available from the stock of materials in seed storage at the Agronomy Farm, K.S.U., for which F_1, P_1, and P_2 seeds could be found in sufficient quantities. Parents selected were the inbreds K41, K724, K201G and H28, and the F_1's (K41 x H28, H28 x K724, and H28 x K201G). The inbred H28 was a common parent to all three F_1 crosses.

Experimental plots consisted of four 20-foot rows, three feet apart, plants 12 inches apart within rows and replicated three times at the North Agronomy Farm. In the summer of 1976, four rows of each F_1 hybrid were planted and flanked on each side by two rows of the parent inbreds designated as P_1 on the left and P_2 on the right. Parental inbreds, K41, K724, K201G, and H28 were also grown in four row plots with three replications. At flowering, all plots containing the parental materials were self-pollinated. The F_1 plots were also self-pollinated. Pollen was collected from the F_1 plots to pollinate the two rows of parental material flanking the F_1 plots on each side. This procedure generated materials for the F_2, BC_1, and BC_2 in one season.

Sampling was done at physiological maturity. Ears selected as samples were obtained by randomly harvesting the required number of ears from each plot. The number of samples taken per plot per replication were five for each inbred (parental line) and 10 for each F_1 entry.

The F_2's - (K41 x H28), (H28 x K724), and (H28 x K201G), and backcrosses - K41 x (K41 x H28), H28 x (K41 x H28), H28 x (H28 x K724), K724 x (H28 x K724), H28 x (H28 x K201G), and

K201G x (H28 x K201G) were produced in the summer of 1977 at the Agronomy North Farm. Entries were grouped according to progeny type, i.e., F_2's, BC_1's, or BC_2's and then randomized within their specific groups. Each entry plot consisted of plants one foot apart in two 30-foot rows, three feet apart, replicated three times.

All entries were self-pollinated and sampling was done at physiological maturity. Due to adverse weather conditions, earworm and cob-rot infestations, the original sampling plan of 20 samples per entry from each replication had to be modified. The modified sampling scheme consisted of harvesting as many good ears as could be found within each entry plot.

C. Laboratory Procedures

a. Sample preparation. After thoroughly washing free of soil and debris, fresh tissue samples (roots, stems, and leaves), were cut into tiny bits to facilitate taking a representative sample and also for easier drying. Samples were placed in an oven and vacuum-dried for 18-20 hours at 50°C. The dried samples were ground through a 20-mesh Wiley mill and pulverized in a wig-L-bug. Powdered samples were wrapped in filter paper and defatted by extraction with Skellysolve B for six hours in Soxhlet extractors. An hour of air-drying was allowed before samples were bottled and labelled appropriately.

Fresh grain samples were prepared differently. After slicing kernals off the cob, five grams of each sample was blended in 120 ml of acetone for one minute. The mixture was

filtered and the residue washed three times with acetone. The use of acetone removed lipids, endosperm coloring and excess water from the fresh grain. The residue was then laid out to air dry for 30 minutes.

Physiologically mature grain samples did not require additional drying. Grinding through a 20-mesh Wiley mill, wig-L-bugging, and defatting with hot Skellysolve B for six hours was the only preparation such samples received.

b. Trypsin inhibitor assay. Assaying enzymatic activity has been simplified significantly by the use of chromogenic substrates. The method used for trypsin inhibitor assay is a modification of the method of Erlanger et al. (17). It calls for use of a direct chromogenic substrate that releases a colored product as a result of enzymic hydrolysis, rather than one requiring a subsequent coupling reaction with a diazonium salt. Experimental search narrowed down to selecting a suitable substrate, an amide, that released a colored amine upon hydrolysis. Benzoyl-DL-arginine p-nitroanilide (BAPA) was selected as the best suitable substrate among several because it released p-nitroaniline which is yellow and can be estimated colorimetrically, and hydrolysed at a rate satisfactory for assaying small quantities of trypsin.

In this study, trypsin inhibitor assay required three reagents, an extracting solution (Tris buffer), the substrate (BAPA) and the enzyme solution (Trypsin). Tris buffer was prepared by dissolving 6.06 g THAM and 2.9 g of $CaCl_2$ (anhyd) in water and diluting to one liter. The pH was adjusted to

8.2, using HCl. For the substrate, 43.5 mg of BAPA were dissolved in 80 ml of H_2O and heated until the BAPA was completely dissolved. This was then diluted to 100 ml with H_2O. For the enzyme solution, 25 mg of trypsin (1-300) was dissolved in 50 ml of 0.001 M. HCl.

Crude extract was obtained by adding 5 ml of 0.05 M. Tris buffer to 0.1 g of defatted sample in a 15 ml centrifuge tube. Centrifuge tubes were rotated on a multipurpose rotator at moderate speed for 30 min. and then centrifuged for another 30 minutes.

Test tubes for enzymic assay were prepared as follows:

Trypsin	Trypsin Reagent Blank	Sample	Sample Blank
2 ml buffer	2 ml buffer	2 ml buffer	2 ml buffer
1 ml H_2O	2 ml H_2O	----------	1 ml H_2O
----------	----------	1 ml extract	1 ml extract
1 ml trypsin	----------	1 ml trypsin	----------

Test tubes were placed in 25°C water bath for 10 minutes. Then five ml of BAPA were added at one min. intervals to each tube. The tubes were mixed thoroughly by shaking and incubated for 10 minutes. Reactions were correspondingly stopped at one min. intervals by adding one ml. of 30% acetic acid to each tube.

Absorbance of the resultant yellow solutions of p-nitroaniline were read at 410 nm on a Beckman Quartz Spectrophotometer. After subtracting the respective blanks, the difference between sample absorbance and that of the stock solution was obtained and expressed as percent inhibition of trypsin.

Percentages were easily transformed into microgram/ml by using a standard curve prepared from isolated corn trypsin inhibitor. Using a multiplier factor of 50, microgram/ml were converted into meaningful microgram/gram of corn.

RESULTS AND DISCUSSION

A. Physiological Study

A trend analysis has been applied to the environmental chamber and greenhouse data. Because the interest here was to determine what differences existed between sampling periods and to identify the direction in which such differences were expressed, a nonparametric test proved a more powerful tool than the most frequently used parametric test. The null hypothesis tested is: all observations within the respective sampling periods do not have any particular order and therefore occur randomly. The alternative is that there is a particular trend to the sampling observations, the direction of which could either be upwards or downwards. Therefore, the null hypothesis was tested against two different alternatives, one for upward trend and one for downward trend.

Accordingly, in a test for upward trend, sampling periods are ordered chronologically 1 to n and the observations also ranked in increasing values 1 to n, irrespective of the period; lower ranks should occur early while higher ranks occur towards the end. Similarly, for the downward trend test, ranking is done from higher to lower values such that ranking is expected to increase over sampling periods, i.e., higher values occur early in the sampling period. On the other hand, rankings for an upward trend analysis are easily converted to rankings for a downward trend analysis by substituting N-Ti for Ti in the

formula, where $N = n + 1$.

The test (30) utilized is $D = \Sigma(i-Ti)^2$ where $i = 1, 2, \ldots,$ n represents sampling order and $Ti = 1, 2, \ldots, n$ is the rank at each particular order. The presence of a trend is indicated by values of D close to or equal to zero. For the reverse trend using the conversion N-Ti, D values are obtained from the relation $D = \Sigma(i - (N-Ti))^2$.

Trypsin inhibitor measurements obtained for germination (Table 1) and greenhouse study (Tables 3, 5, 7) failed to indicate any upward trend (Tables 2, 4, 6, and 8) and were rejected in favor of the null hypothesis of randomness of observations. When tested for a downward trend, only the genotype O_2-S failed to show a downward trend for the germination observations (Table 2). Whole plant and stem observations again were rejected (Tables 4 and 6) in favor of randomness. Inbred line H28 failed to show a downward trend among leaf observations and was rejected (Table 8) in favor of the null hypothesis.

The overall trend exhibited by all eight maize entries indicated by D++ in Tables 2, 4, 6, and 8 is a general decrease in trypsin inhibitor level in the whole plant during the germination period and only in the leaf during the period of vegetative development. There seemed to be no established trend in whole plant and stem samples obtained from the greenhouse study (Tables 4 and 6). The decreasing trypsin inhibitor levels during germination and in leaf during vegetative development have been presented graphically in Figures 1 and 4, respectively. It becomes quite obvious also in Figures 2 and 3 why no trend

Table 1. Observed trypsin inhibitor values (microgram/g) for samples obtained during the germination of eight maize lines.

Sampling period (days)	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
0	684	1099	916	800	836	360	864	901
1	633	1868	883	828	912	284	1021	932
2	728	1258	1077	845	885	208	1168	901
3	609	786	983	1066	1710	224	906	1021
4	524	1634	839	1066	1710	224	906	1021
5	547	1368	596	939	376	76	571	513
6	438	1298	343	563	393	33	482	309
7	291	1002	503	691	296	6	215	278
8	286	1368	320	677	164	44	392	325
9	103	000	309	248	142	22	47	78

Table 2. Ranking and test values for trypsin inhibitor trend analysis of germination data in table 1.

Sampling order (i)	Observation ranking (Ti)#							
	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
1	9	4	8	5	7	10	7	6.5
2	8	10	7	6	9	9	9	9
3	10	5	10	7	8	7	10	6.5
4	7	2	9	10	10	8	8	10
5	5	9	6	8	6	6	6	8
6	6	7.5	5	9	4	5	5	5
7	4	6	3	2	5	3	4	3
8	3	3	4	4	3	1	2	2
9	2	7.5	2	3	2	4	3	4
10	1	1	1	1	1	2	1	1
D_1	329	208.5	312	260	360	318	314	295.5
D_2	9	120.5	18	70	20	12	16	33.5
D^+ = 268.703								
D^{++}= 12.75					5% CRITICAL/D/ = 72.88			

\# = ranking for upward trend

D_1 = test statistic for upward trend = $\Sigma(i-Ti)^2$

D_2 = test statistic for downward trend = $\Sigma\lfloor i-(N-Ti)\rfloor^2$ where N=n+1

D^+ = test statistic for overall upward trend = $\Sigma(i-\overline{T}i)^2$

D^{++}= test statistic for overall downward trend = $\Sigma\lfloor i-(N-\overline{T}i)\rfloor^2$

Figure 1. Trypsin inhibitor level curves of eight maize lines during germination.

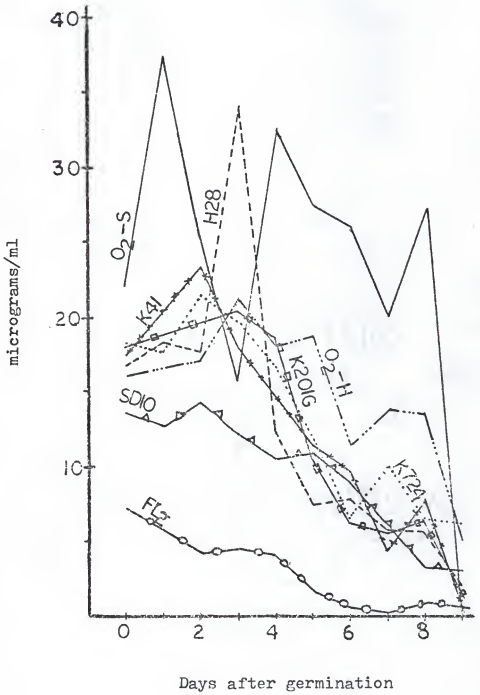


Table 3. Trypsin inhibitor values (micrograms/g) of whole plant samples obtained during greenhouse study of eight maize lines.

Sampling period (days after planting)	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
5	534	1071	277	336	288	64	235	183
7	282	414	327	196	154	223	136	78
11	245	262	317	336	11	452	394	58
14	228	451	287	346	210	308	363	37
16	270	291	402	697	343	441	463	63

Table 4. Ranking and test values for trypsin inhibitor trend analysis of whole plant observations in table 3.#

Sampling order (i)	Observation ranking (Ti)							
	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
1	5	5	1	2.5	4	1	2	5
2	4	3	4	1	2	2	1	2.5
3	2	1	3	2.5	1	5	4	1
4	1	4	2	4	3	3	3	2.5
5	3	2	5	5	5	4	5	4
D_1	34	30	8	3.5	14	6	4	23.5
D_2	6	10	32	35.5	26	34	36	15.5

$D^{+} = 7.47$

$D^{++} = 16.47$ 5% CRITICAL/D/ = 2.66

- footnoting same as table 2.

Figure 2. Trypsin inhibitor level curves of whole plant samples obtained from greenhouse study of eight maize lines.

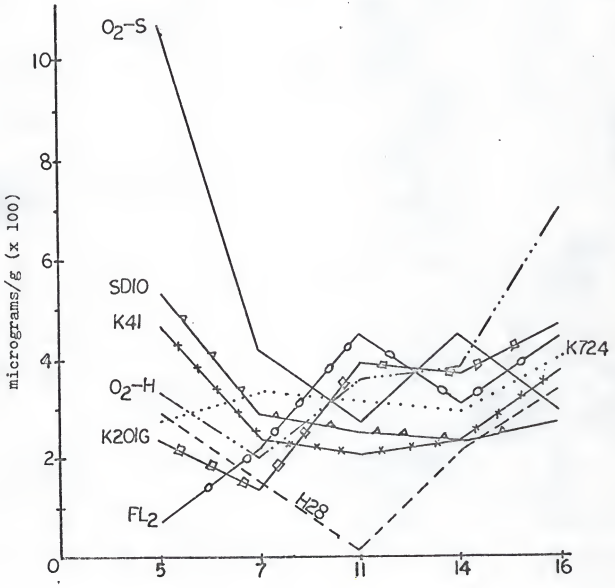


Table 5. Trypsin inhibitor values (microgram/gm) for stem samples obtained during greenhouse study of eight maize lines.

Sampling period (days after planting)	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
26	59	139	54	109	39	106	116	461
32	59	89	000	237	193	122	44	225
42	180	291	74	133	176	69	189	204
53	150	18	292	227	60	54	200	225
63	32	197	000	64	70	80	52	377

Table 6. Ranking and test values for trypsin inhibitor trend analysis of stem observations in table 5.#

Sampling order (i)	Observation ranking (Ti)							
	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
1	2.5	3	3	2	1	4	2	5
2	2.5	2	1.5	5	5	5	1	4
3	5	5	4	3	4	2	4	2
4	4	1	5	4	2	1	5	1
5	1	4	1.5	1	3	3	2	3
D_1	22.5	18	18.5	26	18	32	16	34
D_2	14.5	22	20.5	14	22	8	24	6

D^+ = 14.195

D^{++}= 7.69 5% CRITICAL/D/= 2.66

- footnoting same as table 2.

Figure 3. Trypsin inhibitor level curve of stem samples obtained from greenhouse study of eight maize lines.

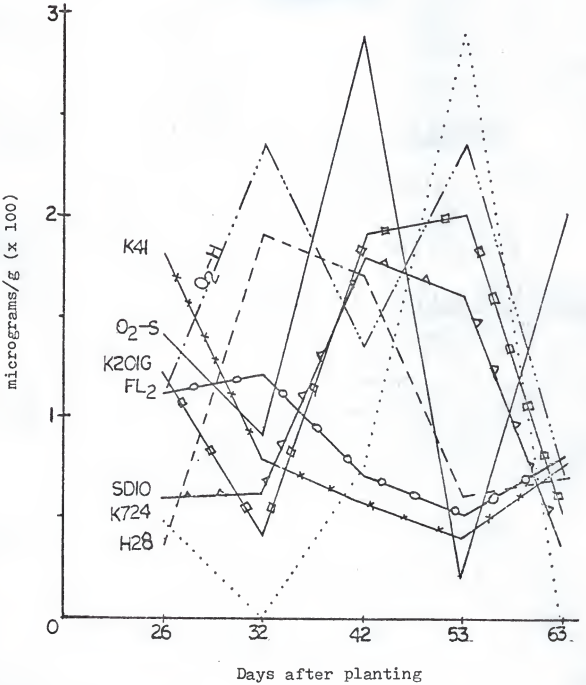


Table 7. Trypsin inhibitor values (micrograms/g) for leaf samples obtained during greenhouse study of eight maize lines.

Sampling period (days after planting)	SD10	O_2-S	K724	O_2-H	H28	FL_2	K201G	K41
26	822	446	227	511	415	595	615	487
32	678	383	276	416	259	382	484	267
42	428	162	166	274	181	148	268	157
53	58	197	206	227	55	69	121	52
63	6	68	10	98	278	144	000	74

Table 8. Ranking and test values for trypsin inhibitor trend analysis of leaf observations in table 7.#

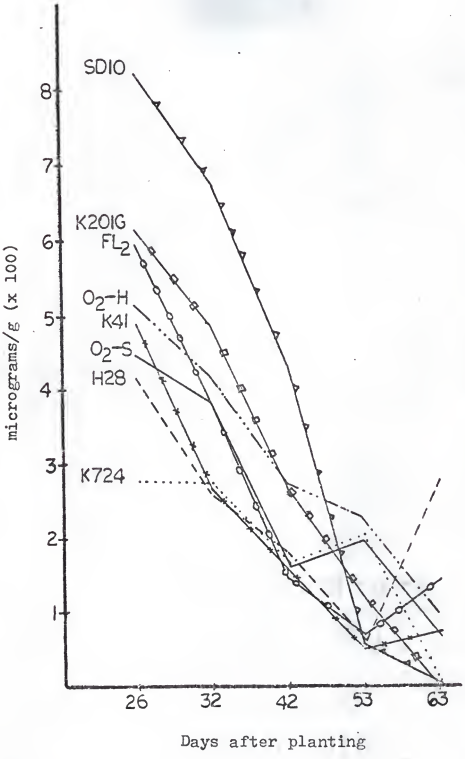
Sampling order (i)	Observation ranking (Ti)							
	SD10	O_2-S	K724	O_2-H	H28	FL2	K201G	K41
1	5	5	5	5	5	5	5	5
2	4	4	4	4	3	4	4	4
3	3	2	2	3	2	3	3	3
4	2	3	3	2	1	1	2	1
5	1	1	1	1	4	2	1	2
D_1	40	38	38	40	28	38	40	38
D_2	0	2	2	0	12	2	0	2

$D^+ = 26.53$

$D^{++} = 0.56$ 5% CRITICAL/D/ = 2.66

- footnoting same as table 2.

Figure 4. Trypsin inhibitor level curves of leaf samples obtained from greenhouse study of eight maize lines.



could be detected in whole plant and stem samples during seedling development when one considers the consistent fluctuation of these graphs from a peak to a trough and vice versa.

A variance analysis on observations taken from flowering to maturity (Table 9) did not show any significant growth stage effect for six of the eight source materials involved in the test. The other two materials, O_2-S and K724 had significant growth stage effects at 5% and 1%, respectively. Significant replication effect for O_2-S and significant location effect for K724 were also indicated by the data.

In light of the observations thereof, there was generally no increase in trypsin inhibitor level during grain filling and maturity. However, the possibility of a change in trypsin inhibitor level during these periods should not be completely ruled out as indicated by O_2-S and K724 responses.

B. Genetic Study

A scaling test has been applied to the relationship between generation means for conformity with the additive-dominance model. To test the adequacy of the model, Mathers formula (34) was used to calculate the quantities A, B, and C and their variances, where

$$A = 2\overline{BC}_1 - \overline{P}_1 - \overline{F}_1 \qquad Var(A) = 4V_{\overline{BC}_1} + V_{\overline{P}_1} + V_{\overline{F}_1}$$

$$B = 2\overline{BC}_2 - \overline{P}_2 - \overline{F}_1 \qquad Var(B) = 4V_{\overline{BC}_2} + V_{\overline{P}_2} + V_{\overline{F}_1}$$

$$C = 4\overline{F}_2 - 2\overline{F}_1 - \overline{P}_1 - \overline{P}_2 \qquad Var(C) = 16V_{\overline{F}_2} + 4V_{\overline{F}_1} + V_{\overline{P}_1} + V_{\overline{P}_2}$$

Table 9. Analysis of variance for postflowering trypsin inhibitor levels for eight maize lines.

Source	DF	SD10		O_2-S		K724		H28	
		MS	F	MS	F	MS	F	MS	F
L	1	65782	2.47	1855	0.03	154393*	9.12	14211	0.15
R	2	21534	0.81	681813*	10.4	33495	1.98	114447	1.23
S	3	34299	1.29	533006*	8.1	451553**	26.7	315429	3.39
L * R	2	408	0.02	11725	0.18	41620	2.46	262994	2.82
L * S	3	73279	2.75	3882	0.06	11924	0.7	423432	4.55
R * S	6	7449	0.28	280902	4.27	22305	1.32	162257	1.74
ERROR	6	26623		65841		16935		93149	

Table 9. (Continued)

Source	DF	H28		FL_2		K201G		K41	
		MS	F	MS	F	MS	F	MS	F
L	1	52267	2.87	11310	0.21	12150	0.61	154240	1.2
R	2	39871	2.19	24105	0.44	37824	1.9	1125	0.01
S	3	44361	2.44	50759	0.93	64822	3.26	355312	2.76
L * R	2	29507	1.62	35140	0.64	17254	0.87	109370	0.85
L * S	3	31787	1.75	43368	0.79	14759	0.74	47740	0.37
R * S	6	8504	0.47	54727	0.9	85082	4.27	53207	0.87
ERROR	6	18183		96417		19904		128806	

* = significant at 5% probability level.

** = significant at 1% probability level.

A, B, and C are expected to equal zero within the limits of sampling error if the model is adequate.

Estimates of the quantities A, B, and C for each cross have been presented in Table 10 along with the generation means for the six populations. In all crosses, at least two of the parameters were significantly different from zero to warrant rejection of the additive-dominance model. A and B were significantly different from zero in H28 x K724, A and C significantly different from zero in H28 x K201G, and B and C significantly different from zero in K41 x H28. The presence of non-allelic interaction can therefore be inferred from the failure to observe the relationships between generation means that were expected on the additive-dominance model by the individual scaling test.

Sewall Wright's formula for estimating gene number as used by Burton (8) has been utilized for estimating the minimum number of genes governing trypsin inhibitor of each cross:

$$n = [0.25(0.75 - h + h^2)D^2]/(VF_2 - VF_1) \quad \text{where,}$$

$$D = \overline{F}_2 - \overline{F}_1 \quad \text{and} \quad h = (\overline{F}_1 - \overline{F}_1)/D$$

The minimum gene number in all three crosses was consistently underestimated that the formula was judged inadequate for this set of data. Values of n obtained were as follows:

$n = 0.07$ for H28 x K724

$n = 0.32$ for H28 x K201G

$n = 0.06$ for K41 x H28

Table 10. Observed means, variance of the mean and scaling test values for six populations of three maize crosses.

Population	H28 x K724		H28 x K201G	
	Observed Mean	Var. Mean	Observed Mean	Var. Mean
P_1	1049.933 ± 48.1477	154.547	1049.933 ± 48.1477	154.547
P_2	974.800 ± 43.7702	127.722	1151.667 ± 103.9659	720.593
F_1	1014.367 ± 76.3807	194.467	769.567 ± 59.8603	119.442
F_2	997.694 ± 188.6818	574.207	1221.689 ± 220.7393	1082.797
BC_1	901.492 ± 156.8250	416.849	989.329 + 171.336	419.372
BC_2	894.000 ± 147.558	426.929	977.148 ± 170.6478	477.388
A	-261.316 ± 44.908*		159.158 ± 44.176*	
B	-201.167 ± 57.183*		33.062 ± 52.436	
C	-62.691 ± 101.23		1146.022 ± 136.666*	

Table 10. (Continued)

Population	K41 x H28	
	Observed Mean	Var. Mean
P_1	1032.333 ± 90.8963	550.809
P_2	1049.933 ± 48.1477	154.547
F_1	919.067 ± 79.9469	213.050
F_2	878.733 ± 232.3931	1200.146
BC_1	962.379 ± 220.8896	841.246
BC_2	826.357 ± 180.0815	579.095
A	-26.642 ± 64.256	
B	-316.286 ± 51.807*	
C	-405.468 ± 144.083*	

* Significantly different from zero.

The formula proved inadequate for the particular set of data because parental selection for each cross was not made on the basis of contrasting trypsin inhibitor levels. It happens to be a basic restriction to the use of this formula, though difficult to find in practice, that parents selected for such a study come from opposite ends of a scale measuring the character - i.e., all the (+) alleles of the n genes, whose differences are involved in the cross, be concentrated in one parental line and all the (-) alleles in the other parental line. Another restriction is the equality of additive effects at all loci such that, in the absence of interaction and linkage, all genes concerned give equal increments. In this study, parents happened to be within such a narrow range of each other in trypsin inhibitor levels that their (+) and (-) alleles were definitely not isodirectionally distributed between the parents. Inequality of additive increment, along with incomplete concentration of like alleles in the parents, must also have contributed to these excessively low estimates of gene number.

Warners method (50) has been used to estimate heritability (h^2). The formula $h^2 = \frac{1}{2}D/VF_2$ estimates heritability in the narrow sense and involves only the F_2 and backcross variances. With D = additive genetic variance and H = dominance variance or portion due to deviations from additivity, the value of $\frac{1}{2}D$ has been obtained from the following relations:

$$VF_2 = \tfrac{1}{2}D + \tfrac{1}{4}H + E$$

$$VBC_1 + VBC_2 = \tfrac{1}{2}D + \tfrac{1}{2}H + 2E$$

Solving simultaneously yields $\frac{1}{2}D = 2VF_2 - (VBC_1 + VBC_2)$.

The heritability estimates obtained have been presented in Table 11. Estimates for H28 x K724 and H28 x K201G were of high values, 69.8% and 80.0%, respectively. That of K41 x H28, 49.6% was of medium value. Total additive genetic variances (D) were higher than total dominance variances (H) in H28 x K724 and H28 x K201G but dominance variance was higher in the cross K41 x H28. All three heritability estimates obtained were significantly different from zero with a mean of 66.4 ± 15.5. The significantly high h^2 values indicate that significant changes in the trypsin inhibitor content in corn can be achieved relatively easily by selection.

Table 11. Heritability estimate (h^2), total additive variance (D), total dominance variance (H) and gene effects for three maize crosses.

	H28 x K724	H28 x K201G	K41 x H28
h^2	0.6976	0.7999	0.496
D	49668.334	77950.028	53583.036
H	27164.496	18709.748	85496.876
m	997.694 ± 23.964	1221 ± 32.906	878.733 ± 34.643
a	7.492 ± 29.048	12.181 ± 29.946	116.022* ± 37.687
d	-397.791* ± 113.258	-1285.035* ± 145.774	-59.526 ± 158.975
aa	-339.792* ± 112.082	-953.802* ± 144.609	62.54 ± 157.746
ad	-30.074 ± 30.238	63.049 ± 33.40	124.821* ± 39.958
dd	862.275* ± 154.104	761.582* ± 181.730	280.388 ± 175.104

* Indicates gene effect significantly deviates from zero.

The various gene effects have been calculated using Gambles method (20) in which

$$m = \overline{F}_2$$
$$a = \overline{BC}_1 - \overline{BC}_2$$
$$d = -\tfrac{1}{2}\overline{P}_1 - \tfrac{1}{2}\overline{P}_2 + \overline{F}_1 - 4\overline{F}_2 + 2\overline{BC}_1 + 2\overline{BC}_2$$
$$aa = 2\overline{BC}_1 + 2\overline{BC}_2 - 4\overline{F}_2$$
$$ad = \overline{BC}_1 - \overline{BC}_2 - \tfrac{1}{2}\overline{P}_1 + \tfrac{1}{2}\overline{P}_2$$
$$dd = \overline{P}_1 + \overline{P}_2 + 2\overline{F}_1 + 4\overline{F}_2 - 4\overline{BC}_1 - 4\overline{BC}_2$$

Additive and dominance gene effects are represented by a and d, respectively; aa, ad, and dd are additive x additive, additive x dominance and dominance x dominance types of epistatic gene effects. Standard errors for testing the significance of the various gene effects were calculated from the variances of the corresponding population means (Table 10).

Gene effects and their standard errors appear in Table 11. In crosses H28 x K724 and H28 x K201G, dominance effects are consistently of a greater magnitude than additive effects and were all negative. Also in the same two crosses, d, aa, and dd were significantly different from zero, confirming the rejection of the additive-dominance model earlier by the individual scaling test.

In cross K41 x H28, additive effect was of a higher magnitude than dominance effect and significantly deviated from zero. Dominance effects were not significant but were again negative. The interaction ad also significantly deviated from zero. The greater magnitudes of the dominance interaction parameters and

their significant deviation from zero suggest evidence for strong non-allelic interacting genes in these crosses.

According to Mather (34), classification of non-allelic interaction is simple when there is only a single pair of interacting genes, but when there are many pairs of interacting genes, only two components, d and dd will reflect the magnitude and signs of the individual d's and dd's, or their balance. The use of a, aa, and ad to explain non-allelic interaction, when more than one pair of interacting genes is suspected, becomes difficult and almost inapplicable without prior knowledge of the magnitudes and signs of the individual a's, aa's, and ad's involved in the a, aa, and ad for all pairs of interacting genes. This difficulty arises from the varying combinations of (+) and (-) signs among the individual gene effects giving the same result in order to create a positive or negative balance. Therefore, any classification of interactions which depends on relative magnitudes and signs will depend on the magnitudes and signs of d and dd in order to be of meaningful interpretation.

In practice, interactions are classified into two types based on the signs of d and dd rather than involving the magnitude since that would require knowledge of the relative magnitudes and signs of the individual d's of the interacting genes. In class type 1, d and dd are of the same sign and therefore considered complementary. In type 2, they are opposite in sign and are referred to as duplicate.

Considering the observations in Table 11, d is significantly negative and dd significantly positive in H28 x K724 and H28 x K201G such that one could say the interactions appear predominantly of the duplicate type.

In K41 x H28 the parameters d and dd are also of opposite signs and fall into the duplicate type of interactions. However, examination of their magnitudes indicates none is significantly different from zero. The effects a and ad were significantly different from zero but no interpretation can be made thereof simply based on their signs.

CONCLUSIONS

Trypsin inhibitor level was traced in corn from germinating seed to grain at maturity. This provided a picture of corn trypsin inhibitor level changes that can be compared to studies with other crops, although it leaves some questions unanswered and raises several others.

The decrease in trypsin inhibitor content of corn seeds during germination parallels findings in peas (26) and mungbeans (3). Whether trypsin inhibitor is degraded like reserve proteins and amino acids translocated to form vegetative proteins in the growing point, or whether it is dissolved slowly to regulate endopeptidase activity during germination, still remains to be investigated.

Fluctuating environmental conditions, viz. humidity and high temperatures, in the greenhouse may have stimulated the influence of physiological factors on level of trypsin inhibitor observed during vegetative development. Since these physiological factors were not studied concurrently with trypsin inhibitor content, they have been ignored for this investigation. In this light, it was concluded that only the leaves showed a trend in trypsin inhibitor level during vegetative development, under normal growth conditions.

During postflowering, no significant difference was noted from one sampling period to the next. The trypsin inhibitor level observed at the blister stage was about the same as that

observed at dough, dent or physiological maturity and it can be concluded that trypsin inhibitor content in the grain must have already been established before the first sample was taken. A more detailed sampling scheme that begins a few days after pollination and involves at least three to four sampling periods within the blister stage, and likewise other stages, might be needed to determine the time of trypsin inhibitor build-up in the grain.

Consistent underestimation of minimum gene number indicated that Sewall Wright's formula was inadequate for the sets of crosses. A look at the parental means, 1049.9 and 974.8, 1049.9 and 1151.7, 1032.3 and 1049.9, for crosses 1, 2, and 3, respectively, supports rejection of the use of the formula based on parents not having contrasting traits; in this instance, one parent not having high trypsin inhibitor levels and the other parent low trypsin inhibitor levels. It is recommended that future research of such nature uses parents based on high and low trypsin inhibitor contents. Even though it might be difficult in practice to find two such parents at opposite ends of a scale, selection of parents with a well-defined range of difference in trypsin inhibitor value might suffice.

Despite the inadequacy of the formula for estimating minimum gene number, it is suggested that gene number, based on the significance of the digenic non-allelic interaction effects, included at least two genes governing the expression of trypsin inhibitor in each of the three crosses.

An attempt to interpret observed heritability values based

on both gene effects and total variances gave a conflicting picture. Dominant gene effects were significantly different from zero in two crosses and additive effects significantly different from zero in the third cross; yet total additive variance was greater in value in the first two crosses, H28 x K724 and H28 x K201G, than total dominant variance. The reverse was true in the cross K41 x H28 in which total dominant variance was larger than total additive variance but additive gene effect, based on the mean approach, was significantly different from zero while dominant gene effect was not significantly different from zero.

Calculation of gene effects based on population means was adequate for the populations on hand but results cannot be projected to future populations since they specifically describe only the populations for which they were computed. Where it is desired to make projections about future generations, the variance approach of calculating gene effects is much more suitable and convenient because inferences about future generations can be made therefrom. This approach, however, could not be applied to the sets of crosses of this investigation due to the lack of an F_3 population which is required by the formula. The use of the variance approach can readily be used to explain heritability values, since heritability is based on total variance values obtained from the variances of the gene effects.

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PHYSIOLOGICAL AND GENETIC STUDIES WITH TRYPSIN INHIBITOR OF CORN (ZEA mays L.)

by

SIZI ZUBAHYEA MORRIS

B.Sc. (Gen. Agric.), University of Liberia, Liberia, 1974

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Trypsin inhibitor, a naturally occurring protein of plants, was studied in corn lines and crosses. The study was in two major parts: 1) a physiological study of eight corn genotypes that traced the level of trypsin inhibitor from germination of the seed, through vegetative development and finally to physiological maturity of the grain and, 2) a genetic study designed to estimate heritability, gene effects, and minimum number of genes involved in the inheritance of trypsin inhibitor in three crosses.

The physiological study, subdivided into a germination, vegetative development and postflowering periods, was conducted in an environmental chamber, a greenhouse, and on the field, respectively. Data obtained for the germination period showed a significant decrease trend for trypsin inhibitor in the seed as days of germination progressed. The greenhouse data, sampled as whole plant, stem, and leaf, depicted a significant decrease for trypsin inhibitor only in the leaf samples during vegetative growth. The field study failed to show any significant difference in trypsin inhibitor level from grain filling to physiological maturity.

The genetic study with 6 populations (P_1, P_2, F_1, F_2, BC_1 and BC_2) of 3 crosses (H28 x K724, H28 x K201G and K41 x H28) was conducted at the North Agronomy Farm, Manhattan.

Minimum gene number was consistenly underestimated and the formula judged inadequate for the sets of data.

Dominance gene effects were significant and much more

important than additive effects in H28 x K724 and H28 x K201G and were of negative values in all three crosses. However, interaction parameters, significant in all three crosses, were of a much higher magnitude than either dominance or additive gene effects and suggested evidence for strong non-allelic interactions in all three crosses. It was found that the non-allelic interactions were all of the duplicate epistasic type.

Heritability estimates obtained were high for H28 x K724 and H28 x K201G (69.76% and 79.99%, respectively) and medium for K41 x H28 (49.6%). All heritability estimates were significantly different from zero. These high estimates suggest that significant changes in the trypsin inhibitor content of corn can be achieved relatively easily through selection.